PEDAL EXPANSION IN THE NATICID SNAILS. II. LABELLING EXPERIMENTS USING INULIN [1]

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The mechanism involved in the hypertrophic dilation of the foot in naticid snails was first correctly explained by Schiemenz (1884, 1887) as being based upon a system of pedal water-sinuses. The peculiar sea-water uptake involved in naticids was then ignored or completely denied by many writers on molluscan mechanics (Lankester, 1883, 1884, 1891; Simroth, 1896–1907) and this continued until relatively recently (Brown, 1964; Morton, 1964; Hyman, 1967). A review of the history of a nineteenth century theory which erroneously attributed distension and protrusion of all kinds of molluscan organs to direct uptake of sea water is provided by Carrière (1882). Russell-Hunter and Russell-Hunter (1968) give a historical survey of published references to Schiemenz's essentially correct conclusions on naticid expansion, review the reasons which contributed to the denial of his work over eighty years, and summarize the present need (in 1968) for an unequivocal demonstration of the use of a pedal water-sinus system in the expansion of naticids.

The work recorded here and in the preceding paper on the naticid water-sinus system was adumbrated in a brief report (Russell Hunter and Apley, 1965) on temporary hyperthermia in *Polinices duplicatus*. Independently, Bernard (1968) and Russell-Hunter and Russell-Hunter (1968) have now reported weighing experiments which demonstrate the nature and extent of the water uptake involved in pedal expansion in naticids. This second paper describes and discusses the use of inulin-labelled sea water in *Polinices duplicatus* in an investigation of the relation of the different water spaces and the rates of water exchange in expanded snails carried out in summer, 1965. A third paper is being prepared on the micro-anatomy and histology of the naticid pedal water-sinus system.

Materials and Methods

Brief notes on the systematics and ecology of naticids are given in the preceding paper (Russell-Hunter and Russell-Hunter, 1968). Only one naticid species was used in the inulin-loading experiments, the more euryoecic and more readily "trainable" form, *Polinices duplicatus*. Medium-sized specimens were employed, all collected by hand from a muddy sand flat at Orleans Town Pond, Cape Cod. For each experimental snail, shell size, contracted weight and mean expanded

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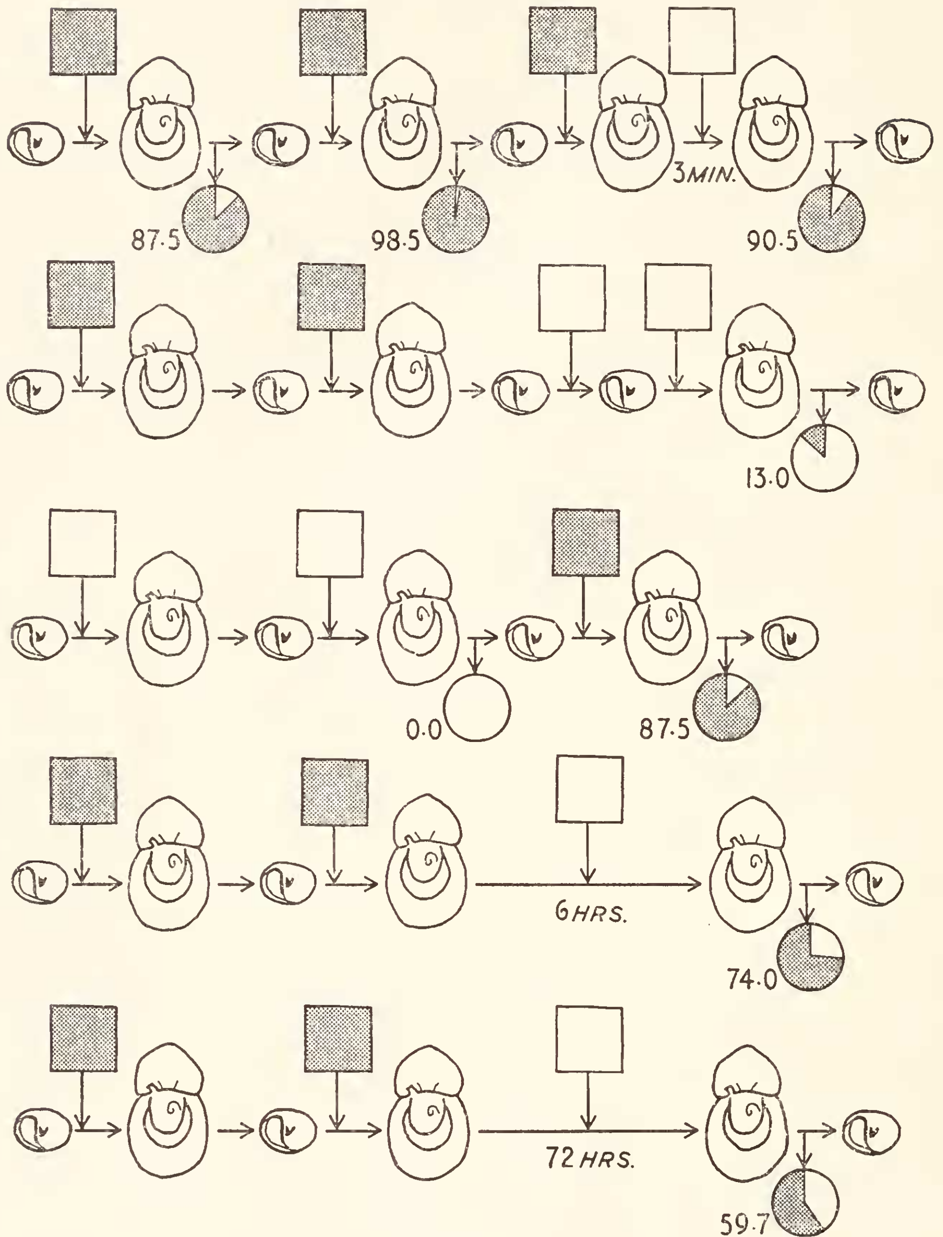

FIGURE 1.

FIGURE 1. Experimental sequences in inulin-labelling of the pedal water-sinus system in the naticid, *Polinices duplicatus,* with average values for some results. The squares above each sequence represent the sea water available for uptake on expansion—shaded squares inulin-labelled, and open squares "clean" sea water; contracted and fully-expanded snails are stylized; and the circles below represent the inulin recovered on forced contraction, expressed as a

weight after training were obtained as in Russell-Hunter and Russell-Hunter (1968). The mean expansion index for each individual allowed a "standard" water uptake to be determined for each, and the actual uptake during an experiment was usually expressed as a percentage of this.

Several sequences were used in inulin-loading, but a typical one would run as follows. A contracted specimen of *Polinices* was placed in a solution of inulin (say 50 mg./l.) in filtered sea water, allowed to expand, forced to contract, allowed to re-expand and then transferred while fully expanded through several washes of clean filtered sea water. It would then be left for a definite time in a known volume of clean sea water, and at the end of the time, weighed in air and forced to contract. Inulin determinations would then be made on the starting solution, the final external sea water, and the internal water expelled at the final forced contraction. This and other sequences are illustrated in Figure 1.

Determinations of inulin concentrations were made by photometric measurement of the red color produced by the reaction of an inulin hydrolysate with resorcinol. Except for minor changes in the preferred range and quantities of samples, this is the method of Schreiner (1950), which in itself is a simplified version of that of Roe *et al.* (1949). Such determinations using resorcinol have been employed by several workers using inulin to determine volumes of extracellular spaces (Ross and Mokotoff, 1951; Cotlove, 1954), and interference from substances likely to be present in biological salines or sea water is minimal. The more cumbersome method using the diphenylamine reaction, employed by some investigators for extracellular volumes (Gaudino and Levitt, 1949), was unsuitable for our purposes.

The analytic procedure (based on Schreiner, 1950) was as follows. Aliquots of 5 ml. of unknown samples or standards were pipetted into 75-ml. Pyrex boiling tubes, each of which already contained 12.5 ml. of 30% hydrochloric acid. Then 5 ml. of an 0.1% solution of resorcinol in 95% alcohol was added to each, and the tubes, loosely stoppered with conical reflux caps, placed in a water bath at 80° C. for 25 minutes. The samples were then cooled in tap water, and the red color determined within 40 minutes. (Trials showed that the absorption values were not significantly changed after 3 hours.) Extinction values were read against a sea-water reagent blank on a Beckman DU spectrophotometer with 1 cm.-path-length cells, mercury lamp and a wave-length setting of 490 mμ. Standards were run with every experiment, as were additional controls (such as samples expelled from the pedal water-sinuses of untreated snails). E-values show a linear relationship to inulin concentrations between 5 mg./l. and 55 mg./l., and experimental procedures were adjusted so that most determinations fell in that range. A stock 0.2% solution of inulin in filtered sea water was prepared at 60° C. (2 g./l.). This was diluted with filtered sea water, immediately before use, to give "exposure"

percentage of the "loading" concentration. These percentages are also shown as figures to the left of each circle and, in three cases, the average elapsed time while expanded in "clean" sea water is also shown. The first sequence is typical of experiments used in investigation of the effects of "multiple loading" and of "flushing" out of the mantle-cavity, the second and third show two methods of investigation of the residual volume of the water-sinus system in contracted snails, and the fourth and fifth typify experiments on the retention of inulin in the water-sinus system over longer elapsed times while expanded. For further explanation, see text and Tables I–V.

solutions in most cases of approximately 50–60 mg. inulin/l. Such high starting concentrations in the experiments with *Polinices* allowed much greater accuracy in the later determinations of inulin in wash waters and in samples from diluted pedal water. Most results are expressed both as concentrations of inulin in mg./l. and as percentages of the (subsequently determined) starting concentration.

Results

In a group of preliminary experiments, numbered and trained specimens of *Polinices duplicatus* (with contracted weights ranging from 17.3 g. to 26.8 g.) were transferred, while contracted, into inulin solutions (46 mg./l. or 97 mg./l.) where they were allowed to expand fully. Thus the water taken into the pedal water-sinuses was inulin-labelled. They were then gently transferred, without much contraction, through successive washes in large volumes of "clean" sea water for periods of from 17 to 34 minutes. After weighing, damp-dried, in air (expanded weights 42.8–67.0 g.), forced contraction yielded water samples (23.5 to 40.6 ml.)

Table I

Inulin concentrations in the pedal water-sinuses of Polinices duplicatus immediately after loading (i. e., less than 1 second washing before force contraction and sampling)

Experiment series #	Snail #	Contracted weight (g.)	Inulin concentration mg./l.		Number of loading cycles	Final concentration as percentage of initial uptake
			Initial uptake	Final sample		
III	R2	26.4	60.0	58.3	3L	97.2
VC	RR8	25.6	63.3	63.0	2L	99.5
VC	PP2	23.8	63.3	61.8	2L	97.6
VC	PP4	29.0	63.3	62.7	2L	99.1
VC	PP9	20.3	63.3	62.8	2L	99.2

from the pedal water-sinuses. These had inulin concentrations ranging from 42.4% to 52.6% of the appropriate starting levels.

This reduction to about half of the starting concentration of inulin in the water recovered from the snails on contraction, obviously could involve several diluting factors. These include: first, residual water present in the contracted water-sinuses before exposure to inulin; secondly, the water contained in the mantle-cavity and rapidly exchanged by the ciliary respiratory currents during the washes in clean sea water; and thirdly, water exchanged between washing baths and the water-sinuses because of small partial contractions and re-expansions during the transfer process. Many of the later experiments were designed to distinguish among these three factors and, as might be expected, the first two proved to be relatively constant for each individual *Polinices*, while the third varied greatly (though contributing least dilution to the results in the best habituated snails).

Before turning to these more complex experiments and to those extending over longer periods of time, it is worth noting that even this preliminary series of results from "single loadings" with inulin is important in confirming the existence of closed spaces containing sea water in the expanded naticid. Recent weighing

TABLE II

Multiple inulin loading of the pedal water-sinuses in Polinices duplicatus, followed by thorough flushing of the mantle-cavity

Experiment series #	Snail #	Contracted weight (g.)	Inulin concentration mg./1.		Number of loading cycles	Final concentration as percentage of initial uptake	Maximum mantle-cavity volume as percentage of water-spaces
			Initial uptake	Final sample			
IIA	R1	23.9	58.3	53.1	4L	91.1	8.9
IIA	R5	16.4	58.3	53.8	4L	92.3	7.7
IIA	P3	26.3	58.3	53.7	4L	92.1	7.9
IIC	R5	16.4	58.3	52.0	4L	89.2	10.8
III	R1	23.9	60.0	57.1	4L	95.2	4.8

experiments on naticids (Bernard, 1968; Russell-Hunter and Russell-Hunter, 1968) merely belatedly confirm and extend Schiemenz's (1884, 1887) findings. The demonstration that a large quantity of inulin-labelled sea water can be carried in the expanded naticid through "clean" wash waters and even through air in a "damp-dried" snail, is a different *kind* of unequivocal evidence for the functioning of the water-sinus system.

In the majority of the 108 successful inulin trials run, the question of the residual volume of the pedal water-sinuses in the contracted snail could be bypassed by multiple loading. In other words the snail was allowed to expand in inulin-labelled sea water, force contracted, then allowed to expand again in the same solution and so on. Initially, three and four successive loadings were carried

TABLE III

Residual volume of the pedal water-sinuses in contracted Polinices duplicatus by two methods

Experiment series #	Snail #	Contracted weight (g.)	Inulin concentration mg./1.		Number of loading cycles	Final concentration as percentage of initial uptake	Residual volume as percentage expanded water-sinus volume
			Initial uptake	Final sample			
VIIC	RR6	36.1	66.8	9.8	2L + 1SW	14.7	14.7*
VIIC	RR1	42.1	66.8	8.8	2L + 1SW	13.2	13.2*
VIIC	PP7	24.8	66.8	8.3	2L + 1SW	12.4	12.4*
VIIC	PP6	24.8	66.8	7.4	2L + 1SW	11.1	11.1*
VIIC	PP2	23.8	66.8	9.0	2L + 1SW	13.5	13.5*
VIIA	PP8	22.5	66.8	59.4	1L	88.9	11.1†
VIIA	PP5	23.5	66.8	57.7	1L	86.4	13.6†
VIIA	PP3	19.5	66.8	59.1	1L	88.5	11.5†
VIIA	PP2	23.8	66.8	57.6	1L	86.2	13.8†

* The first group of five snails were loaded twice with inulin-labelled sea water, force contracted, washed and allowed to re-expand in "clean" sea water before force contracting for the final sample.

† The second group of four snails were taken from clean sea water, force contracted, loaded once with inulin-labelled sea water and rapidly washed in "clean" sea water before force contracting for the final sample.

TABLE IV

Inulin retention in the pedal water-sinuses of Polinices duplicatus after multiple loading and elapsed times from 5 to 20 hours

Experiment series #	Snail #	Contracted weight (g.)	Inulin concentration mg./l.		Number of loading cycles	Elapsed time (hrs. mins.)	Final concentration as percentage of initial uptake
			Initial uptake	Final sample			
VIII	RR1	42.1	61.8	39.8	2L	5.15	64.4
VIII	RR5	35.8	61.8	49.3	2L	5.30	79.8
VIII	RR6	36.1	61.8	50.1	2L	5.31	81.1
VIII	PP8	22.5	61.8	41.5	2L	6.03	67.2
VIII	PP1	28.5	61.8	48.6	2L	6.33	78.6
VB	RR4	36.1	59.6	43.3	3L	12.02	72.7
VB	PP7	24.8	59.6	39.3	3L	12.06	65.9
VB	RR2	41.2	59.6	38.2	2L	12.17	64.1
VA	RR3	30.3	59.9	36.9	3L	20.57	61.6
VA	RR9	24.7	59.9	37.6	4L	20.58	62.8
VA	RR5	35.8	59.9	40.6	4L	21.12	67.8
VA	RR7	33.8	61.1	42.0	4L	21.23	68.7

out, but it became clear that two successive loadings would suffice to label the residual volume (at least within the limits of accuracy imposed by other variables). In Table I are presented five typical results of sampling immediately after multiple loading with only a brief (less than 1 second) wash to remove surface contamination. As can be seen the water expelled from the pedal water-sinuses in these cases has inulin concentrations between 97.2 and 99.5% of the initial uptake. Thus superficial water on the shell and expanded surfaces can only amount to to 0.5–2.8% of the increase in weight of an expanded *Polinices*. In the experiments of Table II, multiple loading was followed by washing, extending over several minutes, in large volumes of sea water. The five cases presented are typical of snails where no contraction or major pedal movement took place during

TABLE V

Inulin retention in the pedal water-sinuses of Polinices duplicatus after longer elapsed times (2–3 days)

Experiment series #	Snail #	Contracted weight (g.)	Inulin concentration mg./l.		Number of loading cycles	Elapsed time (hrs. mins.)	Final concentration as percentage of initial uptake
			Initial uptake	Final sample			
VIB	RR9	24.7	63.1	48.3	2L	58.13	76.5
VIB	PP9	20.3	63.1	40.8	2L	58.11	64.7
VIB	RR8	25.6	63.1	31.2	2L	58.35	49.4
VIB	RR3	30.3	63.1	49.8	2L	58.32	78.9
VIA	PP4	29.0	66.8	33.8	2L	72.15	50.6
VIA	RR7	33.8	66.8	32.8	2L	72.19	49.1
VIA	RR4	36.1	66.8	45.7	2L	72.36	68.5
VIA	RR2	41.2	66.8	47.2	2L	72.34	70.7

the manipulation, and therefore the final sample concentrations reflect dilution by the proportion of water in the mantle-cavity which has had time to become thoroughly flushed out by the ciliary currents. Therefore, the maximum percentage of all water spaces in the expanded snail made up by the pallial cavity lies between 4.8 and 10.8%. Other circumstantial evidence, and an average value of 2% for superficial water, would suggest that for medium-sized *Polinices duplicatus,* mantle-cavity water usually makes from 5 to 7% of the volume taken in on expansion.

The factor of the residual pedal water-sinus volume (when the snail is contracted) is assessed in two ways in Table III. (See also the procedural sequences in Figure 1.) The first group of results in Table III is derived from snails loaded twice with inulin, force contracted and washed while withdrawn, and then allowed to re-expand in "clean" sea water. Thereafter, force contraction yields a water sample from the pedal water-sinuses with an inulin concentration proportional to the residual volume of the water-sinuses when the snail was contracted. The residual volumes thus represent 11.2–14.7% of the volume of the water-sinuses in the fully expanded snail. Another approach was to load snails once only, rapidly wash and then force contract for a water sample. In this case the dilution of the final inulin concentration is proportional to the residual space. Final concentrations of 86.2–88.9% imply residual volumes of 11.1–13.8% by this method—a satisfactory concordance.

As noted above, the majority of experiments began with multiple loading so the potential dilution from the residual volume of the contracted water-sinus (mean value 12.8%) can be ignored. However, all the other experiments on inulin retention in the water-sinuses after various elapsed times can be regarded as showing a basic dilution of up to about 10% resulting from the combined pallial and superficial volumes of water. Superimposed on this are the major differences in water exchange resulting from individual variations in sensitivity and activity which cause different degrees of pedal retraction within the period of the experiment.

Table IV presents some data on inulin retention in snails 6, 12 or 20 hours after transfer to "clean" sea water. Of the water within the pedal water-sinuses, 64.4–81.1% can remain unexchanged for 5–6 hours, and 61.6–68.7% for 21 hours. It should be realized that this set of results represents a biased group, because any snail which has responded to any stimulus by retraction during the 21 hours has been automatically removed from the experimental series.

This bias is even more true of the results on inulin retention over 2–3 days which are presented in Table V. The data were gained from our best habituated snails, and considerable precautions had to be taken to maintain the animals in conditions free from major changes in light, temperature, tactile and vibrational stimuli during the 58 and 72 hours of the experiment. It is remarkable that 49.1–70.7% of the pedal water could remain unexchanged after 72 hours. Other circumstantial evidence, from commensals (Russell-Hunter and Russell-Hunter, 1968), from the occurrence of temporary hyperthermia (Russell Hunter and Apley, 1965), and from other behavioral data, confirms the potentially static nature of the water after it has been taken up into the pedal water-sinuses. It seems likely that, in nature, a large naticid can remain continuously expanded for periods of weeks.

Discussion

Weighing experiments have been used to demonstrate the nature and extent of sea water uptake into the pedal water-sinus system during the expansion of naticids. The experiments with inulin-labelled sea water reported in the present paper provide additional evidence, particularly as regards the relation of the various water spaces and as regards the rates of water exchange. Both topics merit further discussion.

The experiments showed that there are three components in the volume of sea water responsible for the weight increase in expanded snails. Only about 2% is superficial water on the snail's shell and expanded surfaces, and the water rapidly circulating through the mantle-cavity amounts to a further 5–7% of the total. Approximately 90% of the sea-water uptake on expansion enters the pedal water-sinus system. The earlier conclusion that this system is completely separated from the blood in the hemocoelic spaces (Schiemenz, 1887; Russell-Hunter and Russell-Hunter, 1968), is confirmed by the figures for recovery of inulin in many experiments. In other words, there is no dilution of the sea water in the water-sinus system that is not accounted for, and none that could involve exchange with other body fluids. A residual volume of sea water is retained in the contracted water-sinus system and, immediately after contraction, this amounts to 12.8% of the volume of the water-sinuses in the expanded snail. Once again, the evidence from the inulin-loading experiments shows that there is no exchange between the sea water of this residual space and the blood at any time. Similarly, while the snail remains contracted there is no exchange between the residual space and the environmental sea water outside the animal.

Exchange between the environment and the water-sinuses of a fully expanded snail takes place only to the extent that partial contraction followed by re-expansion occurs. (Over a considerable number of "less successful" experiments not fully reported above, there was good correlation between the degree of dilution of the inulin load and the observed frequency and extent of partial contractions.) The data from inulin-labelling prove unequivocally that the pores of the mesopodium remain continuously closed in fully expanded specimens of *Polinices duplicatus*. As noted in the discussion of the preceding paper (Russell-Hunter and Russell-Hunter, 1968), the mechanical value of the pedal sea water to the snail depends on it being closed off from the environment as a hydraulic skeleton (temporarily of constant volume) capable of transmitting forces between antagonistic muscles. It is still remarkable that inulin-labelling showed that 49–71% of the pedal sea water could remain unexchanged after 72 hours.

At first sight, it would seem that surprisingly "stagnant" nature of the water in the pedal water-sinuses would present certain physiological problems to the snail. This does not seem to be so, though questions such as long-term osmotic stress have not yet been investigated. More can be said on the problems of internal temperature and oxygenation. The large static water content, sealed off from the environment, can result in a condition of temporary hyperthermia in *Polinices* (Russell Hunter and Apley, 1965)—a most unusual condition in a marine "poikilothermic" animal. This was detected using thermistor probes in field and laboratory. Snails crawling on exposed tidal flats on a summer's day had internal temperatures ranging from 2.0° C. to above 0.3° C. below the tempera-

ture of the upper 1 cm. of the sand. The temperature of incoming tidal water could be as much as 6.5° C. below the temperature of the sand-flats, and the snails could remain hyperthermic for an appreciable time after tidal submergence. Subjected to lowered temperatures of this order, larger snails (fully expanded weights of 102–159 g.) remained hyperthermic to the extent of 1.8° C. after 5 minutes, 0.9° C. after 10 minutes, and 0.1° C. after 45–60 minutes. Russell Hunter and Apley (1965) note that, empirically, heat is retained about as well as in an equivalent volume of confined but circulating water, but markedly less than in equidimensional foam latex rubber. Such comparison implies some exchange of water *within* the animal during cooling but no exchange with the outside. This is completely in accord with the other evidence on the use of the contained sea water as a hydraulic skeleton. Such a temporary state of hyperthermia has little metabolic importance but is probably of considerable behavioral significance since littoral moon-snails become active immediately after their inundation by the rising tide.

The other physiological question concerns the oxygenation of this "stagnant" mass of internal sea water. There is no evidence to suggest that the water has a lower oxygen tension than that of the snail's blood in the adjacent sinuses. There is circumstantial evidence to the contrary provided by the protistan and harpacticoid commensals which have been recovered from expelled water (Russell-Hunter and Russell-Hunter, 1968). Further, even the most isolated tissues of animals fully expanded for many days remain healthy and responsive. In other words, the ensheathing pavement epithelium and collagen-like connective tissue layers, while mechanically tough, offer no physiological barrier to the diffusion of oxygen.

In broad terms, it seems as if very little energy is expended by a *Polinices* in remaining fully expanded, and perhaps there is a more considerable energy expenditure involved in a complete cycle of contraction and re-expansion. It would seem that the capacity for habituation, which we have exploited in these experiments, must have some adaptive significance. Further, it is clear that sustained contraction and handling over a period of minutes will produce a traumatic change in behavior in *Polinices duplicatus*. "Wild" snails which have experienced such a forced contraction re-expand within 8 minutes, but soon burrow deep into the substratum where they remain immobile but expanded for some time. This behavior was detected as introducing a source of bias when capture-recapture methods were being used to assess population density in *Polinices* (Russell Hunter and Grant, 1966). This study also produced quantitative evidence that recovery from such trauma is complete in just over two tidal cycles, or in about 25.5 hours.

Finally, it should be noted that although inulin-labelling experiments were carried out only on *Polinices duplicatus*, there is reason to believe that the results would be similar with any *large* naticid. All available information suggests that such features as (1) the large proportion (about 90%) of the sea water uptake going into the pedal water-sinuses, and (2) the potentially static nature of the sea water after it has been taken up, would be as true of *Lunatia heros, Polinices lewisi* and *P. josephinus* as they are of *P. duplicatus*.

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Summary

1. Inulin-labelled sea water has been used in an investigation of the pedal water-sinus system, and other water spaces, in *Polinices duplicatus*. Analyses were by a photometric measurement of the reaction of inulin hydrolysate with resorcinol.

2. Of the sea water uptake during expansion: about 90% enters the pedal water-sinus system, about 5–7% is water which rapidly circulates through the mantle-cavity, and about 2% is superficial water on the snail's shell and expanded surfaces.

3. When the snail is in the contracted state a residual volume of sea water is retained in the pedal water-sinus system, and this can amount to 12.8% of the volume of the pedal system in the expanded snail.

4. There is no exchange between the water-sinus system and the blood at any time and, in the fully expanded snail, little or no exchange between the system and the environmental sea water. Labelling showed that 49–71% of the pedal sea water could remain unexchanged after 72 hours.

5. The surprisingly "stagnant" nature of the sea water in the pedal water-sinuses is discussed. Physiological consequences are probably slight, though, under certain ecological conditions, the large static water content is responsible for an unusual condition of temporary hyperthermia. A hypothesis, that little energy is expended by a *Polinices* in remaining fully expanded, is coupled with evidence of traumatic change in behavior resulting from sustained contraction. It seems likely that the features of water spaces and exchange rates demonstrated in *Polinices duplicatus* would be similar in any large naticid.

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THE IDENTIFICATION AND MEASUREMENT OF SUGARS IN THE BLOOD OF THREE SPECIES OF ATLANTIC CRABS [1]

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Although considerable attention has been given to the physiology and biochemistry of the Crustacea, their carbohydrate metabolism did not become known until Hu (1958) found evidence that it was not unlike that of other animals. Using paper chromatography Hu found glucose, maltose, maltotriose, maltotetrose and two other oligosaccharides in the blood of the crab, *Hemigrapsus nudus*. Since then these same sugars have been reported in *Cancer magister* (Meenakshi and Scheer, 1961) several other crabs (Dean and Vernberg, 1965a), the crayfish, *Orconectes virilis* (McWhinnie and Saller, 1960) and the lobster, *Homarus americanus* (Telford, 1968a). The non-reducing disaccharide, trehalose, was found in nine species of crustaceans by Fairbairn (1958) and in traces in some crabs (Dean and Vernberg, 1965a). In the American lobster trehalose appears to be a significant minor component of the blood involved in the response to handling stress (Telford, 1968b). Several monosaccharides, galactose, mannose, fucose and fructose were found with sporadic occurrence (above references).

In this paper the results of chromatographic analysis of the blood sugars in the crabs *Carcinas maenas*, *Cancer borealis* and *Cancer irroratus* are related to measurements of blood glucose and total reducing sugar. Some evidence of changes with the molt cycle and reproductive activity is also given. A comparison is then made between these data and the rather scattered observations in the literature.

Materials and Methods

Collection of animals and blood samples

Both *C. borealis* and *C. irroratus* were obtained from lobster traps at Port Clyde Maine. Three point five to 4.0 ml. of blood was taken via the articular membrane at the base of the cheliped. Decapod crustaceans become hyperglycemic following the stress of handling (Abramowitz *et al.*, 1944). To avoid this reaction blood samples were obtained at the moment of capture with the animals in as nearly an undisturbed state as possible. *Carcinas maenas* was collected in the intertidal zone at Port Clyde. Only from the largest specimens could 4.0 ml. blood be obtained; normally only about 2.0 ml. were collected. The shore crabs, *C. maenas*, were obtained in several stages of the molt cycle but the other two species were taken only in premolt and late postmolt or intermolt because they do not enter the traps at other times. Collections were made monthly from May

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through October, 1966, and September, 1967. The size range of *C. borealis* used was from 6 cm. up to about 18 cm. across the carapace. *Cancer irroratus* is smaller and the range used was 5–15 cm. The size range of *C. maenas* was 4–11 cm. In all three species males are generally larger than females.

As anticoagulant 4.0 ml. Heller's oxalate (Gradwohl, 1943) was used (2.0 ml. for the smaller blood samples). The anticoagulant was evaporated to dryness before use, thus avoiding dilution of the blood sample. The oxalated blood was stored frozen until use.

Preparation of samples

Large amounts of oxalate are necessary because coagulability varies during the molt cycle, presumably because of fluctuating blood calcium levels (Travis, 1955). Excess oxalate, however, interferes with the determination of glucose. Before deproteinizing the 1.0-ml. aliquots of blood, oxalates were precipitated by addition of 2.0 ml. 1% $CaCl_2$. Proteins were precipitated by the Somogyi (1930)

TABLE I

Monthly levels of blood glucose and reducing substances (RS) in Cancer borealis (mg./100 ml.) Port Clyde, Maine, May–October, 1966

Month	♂ N	Glucose	(S.E.)	R.S.	(S.E.)	♀ N	Glucose	(S.E.)	R.S.	(S.E.)
May	48	6.8	(0.49)	10.5	(0.55)	49	8.0	(0.62)	10.0	(0.57)
June	47	7.7	(0.38)	12.2	(0.51)	43	6.6	(0.47)	10.3	(0.54)
July	50	8.0	(0.36)	10.5	(0.45)	43	7.6	(0.25)	9.4	(0.30)
August	51	10.6	(0.47)	15.3	(0.55)	52	8.4	(0.50)	11.1	(0.57)
September	53	10.2	(0.69)	12.4	(0.74)	53	10.2	(0.57)	12.8	(0.63)
October	43	9.9	(0.46)	12.9	(0.52)	52	9.5	(0.45)	12.1	(0.48)
September (1967)	58	8.7	(0.47)	13.1	(0.81)	64	8.8	(0.43)	13.0	(0.67)

method using 2.0 ml. 0.6% NaOH followed by 2.0 ml. 2.2% $ZnSO_4$ (acidified by drop-wise addition of conc. H_2SO_4 so that 1 vol. of zinc sulfate solution exactly neutralized 1 vol. of 0.6% NaOH). After centrifuging a clear supernatant was obtained, representing a 1:7 dilution of the blood. A 1.0-ml. aliquot of this supernatant was diluted 1:4 with distilled water.

Determination of glucose and reducing substances

Glucose was determined in 1.0-ml. duplicates of the original Somogyi supernatant using glucose oxidase-o-diansidine ("Glucostat," Worthington Biochemical Corp., Freehold, N. J.) dissolved in 0.02 *M* phosphate buffer, pH 7.0; further details of this procedure have appeared elsewhere (Telford, 1965). Reducing substances were determined in 1.0-ml. duplicates of the diluted Somogyi supernatant (above) using the Folin-Malmros alkaline potassium ferricyanide method (Dische, 1962). In both cases a blank of distilled water and three glucose standards of 5, 10, 20 mg./100 ml. were prepared in exactly the same way as the blood whenever determinations were made.

Paper chromatography

In preparation for chromatography individual blood samples of 1.0 or 2.0 ml. (as available) were coagulated by brief immersion in a boiling water bath (about 45 sec.). The clot was broken up and extracted with three washings of 60% methanol which were then pooled. Deionization on ion exchange columns followed (Telford, 1965) and the eluate was dried at 40° C. The residue was redissolved in 0.1 or 0.2 ml. pyridine (depending on size of original blood sample). Various sugars, alone and in mixtures, were prepared in the same way without showing any changes in chromatographic characteristics. The pyridine solutions were spotted onto Whatman #3 paper and developed for 16 hr. in a descending flow of ethyl acetate:pyridine:water, 8:2:1 (v/v/v) (Jermyn and Isherwood, 1949).

Detection of sugars on the developed chromatograms was by one of three

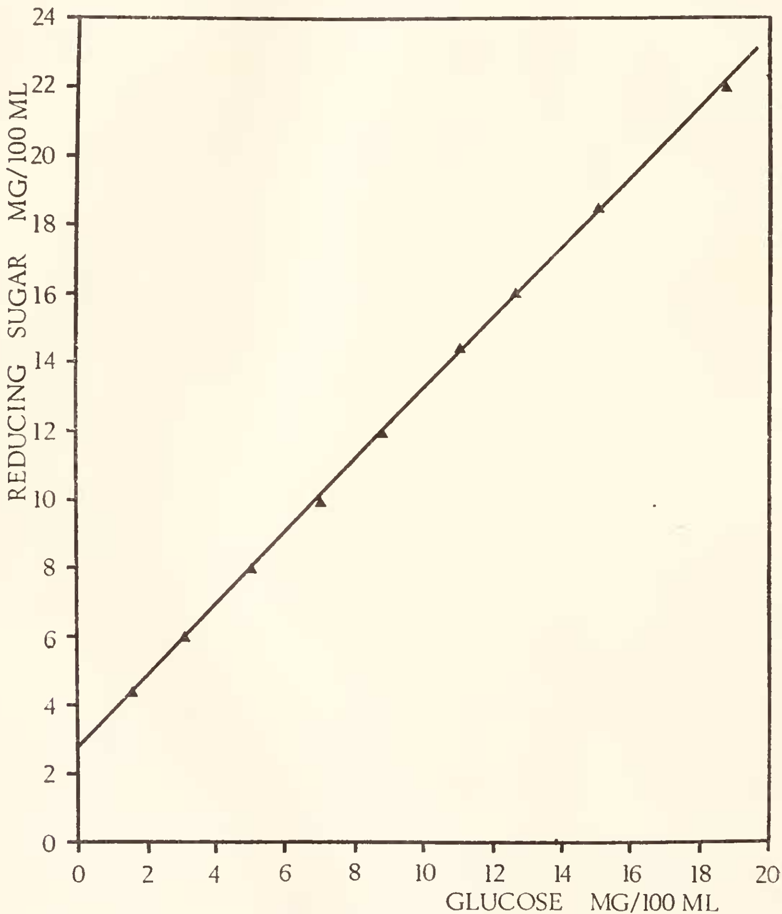

FIGURE 1. Relationship between blood glucose and reducing substances in the crab, *Cancer borealis*.

ways: (1) spray with 3% phthalic acid in 95% ethanol, viewed under UV light, (2) 0.5% benzidine in acetic acid-trichloracetic acid-ethanol, and (3) $AgNO_3$ in acetone followed by 10% NaOH in 80% methanol (all methods in Dawson *et al.*, 1959).

Estimates of concentration in the spots were made by visual comparison with spots of known concentration and by chromatographing various sizes of samples to determine threshold of visibility with the detecting agents. Neither method is particularly accurate but estimates of the right order of magnitude are obtainable.

Reagents

The chemicals used were Fisher "Certified" and the carbohydrates for chromatography were obtained from Sigma Chemical Co. Ltd.

Results

Mean levels of glucose and total reducing substances (RS) in the blood of *C. borealis* made at monthly intervals are given in Table I, together with the number of specimens (N) and the standard error of the mean (S.E.).

Table II

Blood glucose and reducing substances during the molt cycle of Carcinas maenas (mg./100 ml.)

Stage	Drach (1939)	N	Glucose	S.E.	Reducing sugar	S.E.
Intermolt	C_4–D_0	23	9.9	(0.81)	13.8	(0.72)
Premolt	D_1–D_4	11	12.2	(1.37)	17.1	(1.41)
Very soft	A_1–A_2	5	5.8	—	7.9	—
Early postmolt	B_1–B_2	2	5.7	—	8.0	—
Postmolt	C_1–C_3	19	8.4	(0.90)	13.5	(1.07)

An analysis of variance indicates that the variation in mean monthly glucose levels (1966) is significant at the 0.05 level. The differences in mean monthly reducing substance levels are generally not significant although a t-test between the July and August levels in males gives a significant result. In spite of appearances the difference between the sexes is not significant.

The individual glucose and reducing substance levels varied quite widely. Glucose ranged from 0.4 mg./100 ml. to 29.3 mg./100 ml. and the range for reducing substances was 3.3–29.5 mg./100 ml. The relationship between glucose and reducing substance is approximately constant. Figure 1 is a plot of mean glucose levels within 2 mg./100 ml. classes against the corresponding mean reducing substance levels. The line has a slope of 1.02 and an intercept of 2.9 mg./100 ml. when glucose is nil. The same values for slope and intercept can be obtained directly. The correlation coefficient between glucose and reducing substances, calculated directly from the 584 paired measurements, is highly significant ($R = 0.914$, $t = 54.22$ with 582 degrees of freedom). The analysis of covariance shows that this relationship between glucose and reducing substances

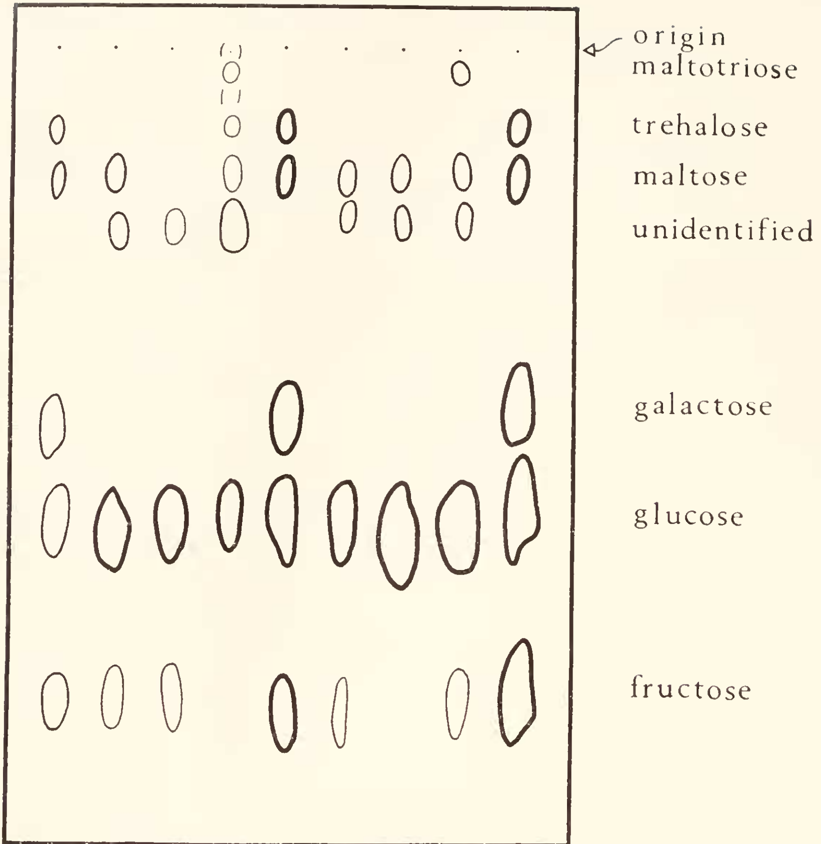

FIGURE 2. Paper chromatogram of blood sugars of *Cancer borealis.* Left, center and right, control mixtures of trehalose, maltose, galactose, glucose and fructose, 1.5 γ, 3.0 γ and 7.5 γ each, respectively (traced from photograph).

does not vary significantly from month to month, nor between the sexes, and it indicates the same level of significance for the variations in monthly mean glucose and reducing substance levels as did the analysis of variance already cited.

The crabs, *C. maenas,* were collected in September, 1967, in various molt stages. Intermolt and very early premolt, $C_4 - D_0$ of Drach (1939), were not separated but were treated together as a single intermolt stage which was recognized by color, hardness of shell, texture, color and consistency of the blood etc. Premolt, stages $D_1 - D_4$ of Drach, was recognized by darker color with blueness of chelipeds, development of new epicuticle and exocuticle and regeneration of damaged appendages. The earliest postmolt stage, stages $A_1 - A_2$ of Drach, was easily recognized by the non-calcified shells and the next postmolt stage, stages $B_1 - B_2$ of Drach, by the partially calcified exoskeleton. Postmolt, $C_1 - C_3$ of Drach, was recognized by the color and texture of the shell and blood. Table II shows blood glucose and reducing sugars in these general molt stages. These dif-

ferences are found by analysis of variance to be significant at the 0.05 level. No attempt was made to determine the significance, if any, of the differences between sexes, because there are too few observations to produce a reliable result.

A total of 67 specimens of *C. irroratus* were taken from lobster traps in May and June (1966), 31 males and 36 females. The mean glucose level for these was 8.1 mg./100 ml. (S.E. 0.23) and reducing substances 11.9 (S.E. 0.39); no difference in sex was apparent. No other determinations were made on this species (except chromatography, below) because *C. borealis* was readily available in much greater numbers in the areas where lobster traps were being set.

Chromatography of deionized blood samples (Figs. 2 and 3) revealed the occurrence of six sugars in the amounts estimated in Table III. Mobility of the sugars is expressed relative to glucose (Rg) because the solvent front was allowed to leave the paper. The bracketed percentage figure indicates the approximate number of samples in which each sugar was found. The estimated quantity is maximal in these samples. The unidentified carbohydrate was com-

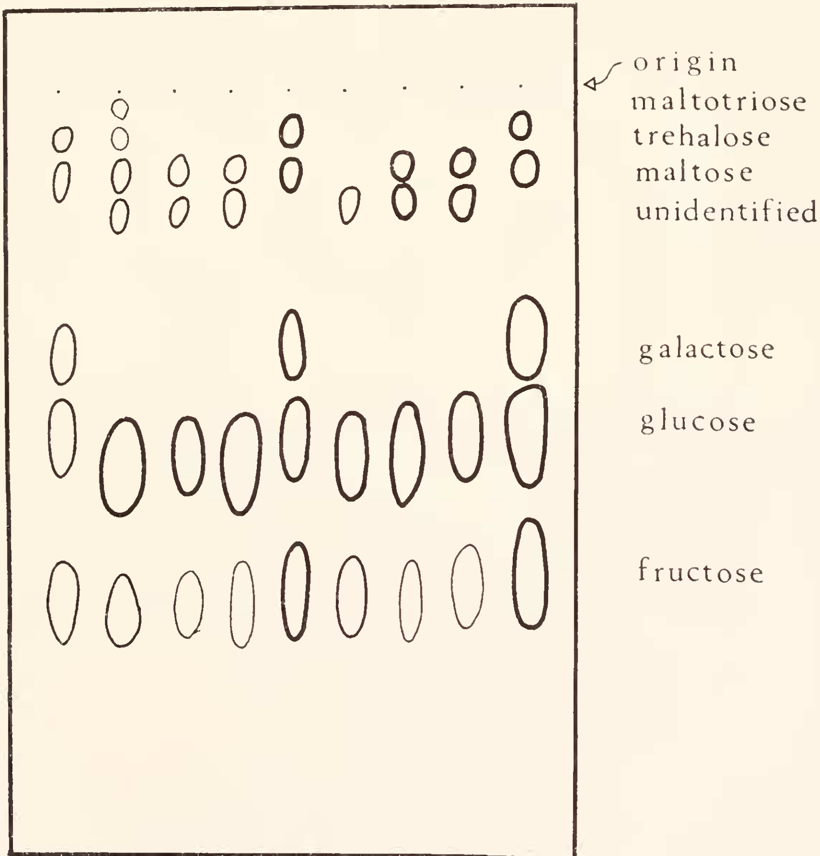

FIGURE 3. Paper chromatogram of blood sugars of *Carcinas maenas*. Controls as in Figure 2 (traced from photograph).

pared to maltose in estimating its concentration. The following criteria were used for identification of the sugars. Maltotriose: plot of $\log_{10}$ Rg against number of hexose units places it on a straight line with maltose and glucose (McWhinne and Saller, 1960) (Fig. 4). No maltotriose was available for direct comparison. Trehalose: same mobility as control spots, reinforcement of spots when trehalose added to sample, lack of reaction with phthalic acid or benzidine indicates non-reducing nature, appearance of spot with silver nitrate at same time as trehalose (this is due to the very strong alkali used, 10% NaOH). Maltose, glucose and fructose: mobilities, lack of proliferation when these sugars were added to blood samples, corresponding blood sugars show reducing activity and appear in same sequence and time intervals with $AgNO_3$-NaOH spray (fructose-glucose-maltose). The mobility of the unidentified component suggests a disaccharide and its reaction with both phthalic acid and benzidine indicates a reducing group.

A total of ten female *C. borealis* was obtained carrying egg masses. On one of these the sponge was old; blood glucose and reducing substances levels were

Table III

Estimated concentrations of blood sugars found in three species of crabs with the frequencies of occurrence (%)

Sugar	Rg	*C. borealis*	*C. irroratus*	*C. maenas*
Maltotriose	(0.05)	trace (10%)	trace (30%)	2 mg./100 ml. (30%)
Trehalose	(0.13)	trace (10%)	trace (10%)	1.5 mg./100 ml. (10%)
Maltose	(0.27)	2 mg./100 ml. (70%+)	2 mg./100 ml. (70%)	3 mg./100 ml. (30%)
Unidentified	(0.32)	5 mg./100 ml. (100%)	5 mg./100 ml. (100%)	5 mg./100 ml. (100%)
Glucose	(1.00)	— (100%)	— (100%)	— (100%)
Fructose	(1.40)	1 mg./100 ml. (60%)	1 mg./100 ml. (30%)	1.5 mg./100 ml. (100%)

8.0 and 8.2 mg./100 ml., respectively, in this individual. The respective mean levels for the remaining nine were 12.36 (S.E. 1.18) and 14.47 (S.E. 1.19) mg./100 ml. Twelve females were found with bright orange-red blood and well developed ovaries. These were presumed to be preparing for spawning since the blood color was closely similar to that of the eggs and ovaries and this coloration never appeared in males. Mean glucose and reducing levels were 11.7 (S.E. 1.28) and 12.5 (S.E. 1.17) mg./100 ml. Three pairs of *C. maenas* were captured in copulation and blood was taken from all of the males and two females. No significant difference in blood sugar levels was found despite the ire and pugnacity of the animals (glucose 10.3, reducing substances 13.9 mg./100 ml.).

Discussion

The blood glucose and reducing sugar levels reported here are comparable with those reported previously for decapod crustaceans but some interesting differences emerge. McWhinnie and Saller (1960) found in the crayfish *Orconectes virilis* that glucose levels averaged 3–4 mg./100 ml. and made up about 20–25% of the total reducing substances (by Folin-Wu method). Earlier McWhinnie and Scheer (1958) found the same relationship between glucose and total carbohydrate

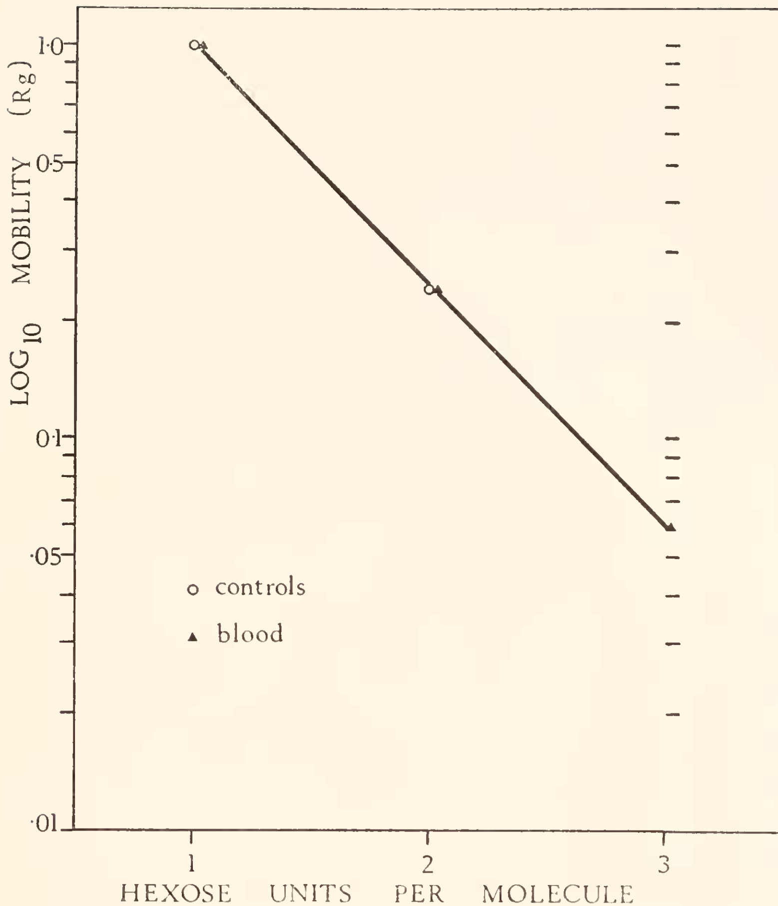

FIGURE 4. Identification of presumed maltotriose by plotting molecular size and $\log_{10}$ mobility (after McWhinnie and Saller, 1960).

(by anthrone method) in the crab *Hemigrapsus nudus.* More recently Dean and Vernberg (1965a, b) using the same methods as McWhinnie and Saller (1960), found the same relationship in several species of crabs and stated specifically that no matter what the glucose level, its ratio to total reducing substances was constant. This is clearly not the case in *C. borealis* where glucose is the variable blood sugar and the non-glucose component remains approximately constant at about 3 mg./100 ml. In the lobster, *Homarus americanus,* this same relationship has been found, variable glucose levels and approximately constant non-glucose around 4.4 mg/100 ml. (Telford, 1968a). In the special case of females carrying eggs there is evidence of significant differences between them and other females (0.01 level by *t*-test). In Table IV these and similar data from Dean and Vernberg (1965b) are compared.

A similar series of changes in glucose levels evidently occurs in the two species but according to the data for *C. sapidus* another reducing substance also undergoes

the same changes. No attempt was made to identify this substance. Dean and Vernberg (1965b) appear to have used a heat-coagulated blood for their reducing substance determination. This method does not completely deproteinize the sample and leaves most or all of the non-carbohydrate reducing matter in the filtrate. These interfering substances may have masked the true relationship between glucose and reducing sugars in the studies with heat-coagulated blood.

The amount of color produced by several sugars with the Folin-Malmros method used in this study was determined and expressed as a percentage of the color given by the same amount of glucose. Trehalose, as expected, gave no reaction; maltose gave 60% of its glucose equivalent, fructose 104%. The estimated amounts of sugars other than glucose found in chromatograms (Table III) are the maximum levels found. Assuming that the unknown reducing disaccharide would react like maltose (lactose, for example, gives about 55%), then the total non-glucose carbohydrate component of the reducing substances would have a maximum value of about 6 mg./100 ml. The estimated normal level is closer to 2 mg./100 ml. or,

Table IV

Glucose and reducing substances levels in C. borealis and C. sapidus carrying egg sponges

Species	N	Glucose mg./100 ml.	S.E.	Reducing mg./100 ml.	S.E.	Gluc/Red × 100
Cancer borealis						
no eggs	43	6.59	(0.47)	10.31	(0.55)	64%
new eggs	9	12.36	(1.18)	14.47	(1.19)	85%
old eggs	1	8.00	—	8.20	—	97%
Callinectes sapidus						
no eggs	7	18.47	(2.36)	—	—	20–25%
new eggs	7	37.61	(2.77)	—	—	20–25%
old eggs	9	10.52	(1.03)	—	—	20–25%
C. sapidus data from Dean and Vernberg (1965b)						

about 60–65% of the non-glucose component. In another study of the lobster, *Homarus americanus,* the non-glucose part of the reducing substances was estimated to be about 50% carbohydrate (Telford, 1968a, 1968b).

In the study of the lobster referred to above a cycle of changes in blood sugar levels during the molt cycle was found in a group of 800 animals. The apparent cycle found here in *C. maenas,* although the number of specimens is much lower, is closely similar. Molting of crabs on the coast of Maine occurs principally in the late summer and fall. The seasonal changes in *C. borealis* probably reflect the molting cycle with glucose increasing in premolt (August-September) and dropping in postmolt. The differences here are not clearly defined because the different molt stages were not separated, the monthly samples being made up of a changing proportion of premolt and other animals. In previous studies such a cycle of changes has not been found although expected (McWhinnie and Scheer, 1958; Scheer, 1959; McWhinnie and Saller, 1960).

As a regular component of crustacean blood fructose has not previously been reported. It was reported as occasionally occurring in *H. americanus* (Telford,

1965; 1968a, 1968b) but it was not found by either McWhinnie and Saller (1960) or Dean and Vernberg (1965a). It has been reported in some insects (Levenbrook, 1950). No sign of the curious "galactan derivative" of McWhinnie and Saller (1960) was found, nor of galactose. Maltotetrose has often been reported by other workers but no spot corresponding with its probable position could be found with the detection reagents used here, nor by examination under UV light when tetroses should fluoresce. Following molting the blood of *C. maenas* has only traces of sugars other than glucose; the oligosaccharides are severely depleted as in lobsters at this stage (Telford, 1968a).

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Summary

1. Blood glucose levels in the crab *Cancer borealis* ranged from 0.4 mg./100 ml. to 29.3 mg./100 ml. with a mean value of 8.6 mg./100 ml. In the same animals blood reducing substances were in the range 3.3–29.5 mg./100 ml. with a mean of 11.6 mg./100 ml. Blood glucose and reducing substances in the other two species of crabs tested, *Cancer irroratus* and *Carcinas maenas,* were in the same ranges.

2. Changes in the blood glucose level account for most of the variations in reducing substances; the other components remain approximately constant, at about 3 mg./100 ml. The relationship between blood glucose and reducing substances is thus a simple straight line one.

3. Variations of blood sugar levels during the molt cycle were found in *Carcinas maenas* and probably occur also in *Cancer borealis.* Qualitative changes in blood sugar composition also occur.

4. Significant changes also occur in *Cancer borealis* females carrying eggs and at this time the relationship between blood glucose and reducing substances changes, the two values converging as the egg mass ages.

5. Paper chromatography of the blood of these three species of crabs shows the presence of glucose, fructose, maltose, an unidentified reducing disaccharide and occasional traces of maltotriose and trehalose.

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